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Changes in the human plasma and urinary metabolome associated with acute dietary exposure to sucrose and the identification of potential biomarkers of sucrose intake.

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KEYWORDS

Metabolomics, sucrose, erythronic acid, metabolism, biomarkers

ABBREVIATIONS

CHO, carbohydrate; DF, Discriminant Function; FIE-MS, Flow-Injection Electrospray Mass Spectrometry; GC-tof- MS, Gas Chromatography –time-of-flight - Mass Spectrometry; FT-icr-MS, Fourier Transform-Ion Cyclotron Resonance Ultra-Mass-Spectrometry; PC-LDA, Principal Component Linear Discriminant Analysis; RF, Random Forest.

Abstract

Scope: The intake of sucrose is of public health concern but limited information is available on the metabolic effects of short term exposure. Our aim was to use metabolomics to investigate the metabolic impact of acute sucrose exposure.

Methods and results: We performed a randomised, parallel, single-dose feeding study on healthy females (n=90, aged 29.9 ± 4.7 yr, BMI 23.3 ± 2.5 kg/m²) consuming either 0g, 50g or 100g sucrose in 500ml water. Blood and urine samples were taken before and for 24 h post sucrose intake. Urine and plasma samples underwent detailed metabolite profiling analysis using established protocols. FIE-MS fingerprinting analysis showed that 3 h after intake was the most informative time point in urine and plasma and out of 120 explanatory signals, highlighted 16 major metabolite signals in urine and 25 metabolite signals in plasma that were discriminatory and correlated with sucrose intake over time. The main confirmed metabolites positively correlated with intake were sucrose, fructose and erythronic acid, while those negatively correlating with intake included fatty acids and derivatives, acyl-carnitines and ketone bodies. GC-tof-MS profiling analysis confirmed the fingerprinting data.

Conclusions: Acute exposure to sucrose identified a number of metabolites correlated with sucrose intake and several compounds attributed to metabolic fasting.

1. Introduction

Carbohydrates are an important source of energy in the UK population with the average contribution to total food energy intake being approximately 48% for adults [1] and a current recommendation of 50%. Sucrose is generally obtained from sweetened snacks and beverages but a small portion can also be obtained from intrinsic sources such as fruits & vegetables [1]. Current average intakes for extrinsic sugars in the UK are 72 g/d and 52 g/d for adult men and women, respectively. Recently it is becoming clear that excessive intakes of rapidly absorbed added sugars and sugar-sweetened snacks are of health concern and

related to negative health effects such as insulin resistance (IR), type 2 diabetes, obesity, cardiovascular disease and dental caries [2, 3].

Recent research has looked at the metabolic consequences of consuming sucrose as whole, or its digestion products fructose and glucose [4-6]. Increased fructose and or sucrose intake can induce a hyperinsulinaemic state that has prolonged effects on lipid metabolism [7, 8]. These studies have shown that fructose consumption, but not glucose consumption, increased plasma triglyceride concentrations, increased de novo hepatic lipogenesis, increased concentration of smaller dense LDL, and decreased lipoprotein lipase activity. Fructose consumption also increased fasting glucose concentrations and decreased insulin sensitivity [5, 6, 8]. In studies investigating the effects of glucose on metabolism during an oral glucose tolerance test, metabolic changes to a number of pathways in central metabolism including proteolysis, lipolysis, ketogenesis and glycolysis were revealed [9-11]. It is clear then that consuming sucrose has profound metabolic effects and is of interest to investigate.

Following sucrose intake it has been shown that there is a time-dependent appearance of fructose and sucrose in the urine [12] and urinary sucrose and fructose have been suggested as predictive biomarkers for sucrose intake [12-15], which has been recently reviewed [16]. Luceri et al [12] found a significant correlation between sucrose intake and both sucrose excretion and fructose excretion in spot collections and Tasevska et al [13] found urinary excretion of sucrose and fructose to be significantly correlated with sucrose intake in daily 24 h urine collections under highly controlled conditions, such that 200 g of total sugars intake predicted 100 mg sucrose and fructose in the urine [13]. More recently, the same authors found that self-reported sugar intake was significantly misreported when using urinary sucrose as a biomarker in a larger population [17]. However, although a relationship between urinary sucrose excretion and extrinsic sugar intake clearly exists, its use in free-living population studies requires calibration equations [18], and it is not clear if this method will be able to quantitatively determine intake of these sugars in free-living populations [13, 16] and other potential biomarkers of sucrose intake would be interesting to develop.

There has been an interest in developing metabolomic approaches to monitor dietary intake and their effects on metabolism [19, 20]. By virtue of its comprehensive and non-selective approach to sample analysis, metabolomic procedures can define metabolic signals indicative of dietary exposure not previously considered, and has offered the potential of developing biological markers of dietary intake [20-22].

In the current study we have used standardized protocols for metabolite fingerprinting of spot urine and plasma samples [21, 23] to investigate the influence of acute sucrose intake on metabolite profiles. Our aims overlap and are two-fold; to use metabolomics to firstly explore the specific metabolic impact of acute sucrose exposure and secondly to identify metabolites that are correlated to sucrose intake that offer potential to be further developed into quantitative biomarkers. These are important to establish given the public health interest in sucrose intake and to interpret diet-disease associations in population studies.

2. Materials & Methods

2.1 Ethical approval and subject recruitment

The procedures used were in accordance with the Helsinki Declaration of 1975 as revised in 1983. The project was granted ethical approval by the Cambridgeshire 3 Research Ethics Committee (ref: 09/H0306/56). Subjects were recruited at two centres, Cranfield University and HNR-MRC at Cambridge through advertisement and from previous study databases. Volunteers were initially interviewed by phone and assessed for suitability via a screening questionnaire relating to their general health and carbohydrate intake.

Inclusion criteria were female, outwardly healthy and free from prescription medication, non-smoking, aged between 25-40, and a BMI between 20 – 30 kg/m². Exclusion criteria included extremes of CHO intake (estimated at <30% or >60% of energy from food diaries and specific dietary questions), those taking nutritional supplements in preceding 2 months, being pregnant, history of any metabolic disease, recent use of hypolipidemic therapy, systemic corticosteroids, or those receiving drugs for regulating haemostasis or chronic disease, weight change of >3kg in preceding 2 months, unwilling to follow the protocol and/or give informed consent were also excluded.

2.2 Study design

A randomised, parallel, controlled study, to compare the response of subjects (n=30 per group) consuming a test drink containing either 0, 50 or 100 g sucrose in water followed by sampling of blood at 0, 3 and 24 h, and urine at 0, 3, 6, 9 and 24 h. We aimed for 30 individuals for each treatment, 90 were required in total.

Subjects were provided with standard meals on the evening prior to the study day, at lunch, and on the evening of the study day in order to reduce major fluctuations in carbohydrate intake and decrease metabolite variability. Each meal contained a relatively low content of carbohydrate and no extrinsic sugars. Foods were purchased in bulk from the same supermarket to avoid any availability issues. Subjects avoided food & beverages containing sucrose for the study period from day 0 until when the 24 h samples were taken.

On the evening prior to the study, subjects emptied their bladder before eating a standard meal of baked salmon fillets with steamed vegetables (carrots, cauliflower and broccoli) (719 kcal, 68.6 g protein, 45.9 g fat, 9.1 g CHO with 3.6 g as sugars) and collected their urine into a suitable container for the rest of the evening, overnight and first void in the morning (1st overnight, ON1). Urine was stored in a cool bag with cool packs. Subjects fasted for a minimum of 12 hours. On the study day, subjects reported to the Nutrition Unit (at either Cranfield or HNR-Cambridge) where they had anthropometric, body composition and blood pressure measurements taken and provided a spot urine collection (0 h fasting spot). Immediately after subjects provided a fasting baseline blood sample (0 h fasting blood) they were given their test drink of 0, 50 or 100 g sucrose in 500 ml water (randomized groups A, B and C, respectively). Water was provided by the investigators on the study day (2 x 500 ml) and the subjects were encouraged to drink throughout the day. At 3 h post consumption the subjects were again asked to provide a spot urine collection (3 h spot) and again immediately afterwards subjects provided a blood sample (3 h blood). Subjects were provided with a standard lunch of baked haddock with steamed vegetables (as above) (496 kcal, 46.8 g protein, 28.1 g fat, 14.1 g CHO with 6.9 g as sugars). Further spot urine collections were taken at 6 hours post (6 h spot), and 9 hours post (9 h spot). Subjects were provided with a standard evening meal of grilled chicken with steamed vegetables (as above) (599 kcal, 46.6 g protein, 39.1 g fat, 13.8 g CHO with 4.4 g as sugars), before which they emptied their bladder and collected their urine for the rest of the evening, overnight and first void in the morning (2nd overnight, ON2). Subjects then fasted for a minimum of 12 hours and were not allowed any further food or drink (apart from water). Subjects then reported to the Nutrition unit at the same time as for the previous day, provided a further fasting spot urine collection (24 h fasting spot) and a blood sample (24 h blood). No other food and drink, other than that provided by the investigators, was consumed during the study period.

2.3 Anthropometric measurements and Dietary analysis

Height was measured using a wall mounted Stadiometer. A Tanita™ segmental body composition analyser (BC-418) was used to assess body composition (% body fat, BMI and weight) immediately after measurements of height. Subjects removed their socks and shoes prior to recording and a researcher ensured that the subjects were standing correctly on the pads and holding the handheld pads required for full assessment. Measurements were taken on the morning of the study day in a fasted state. Blood pressure was measured using a handheld electronic device. Subjects were allowed to rest for 5 minutes before measurements were taken and triplicate measurements were taken over a 5 minute period and averaged. Subjects were monitored for their habitual food intake prior to the trial using a 4-day food diary which ended on, and included, the day before the trial (day 0). The food diary was pre-validated, developed by the MRC-HNR at Cambridge. The food diaries were converted to macro and micronutrient intake using the Dietplan 6 software package (Forestfield Software 2008), which utilizes food composition tables from McCance and Widdowson [24], and with additional information from food manufacturers and recipe analysis. Dietary analysis was co-ordinated and performed by the dietetic group in the Division of Nutritional Sciences, University of Surrey.

2.4 Biological sample collection

Blood was taken by a suitably trained person using the Sarstedt Monovette system. On each occasion blood was drawn into 2 x 8 ml lithium heparin (Li/Hep) tubes and centrifuged (3500 rpm at 10°C for 15 min) exactly 15 min following blood collection. Aliquots of plasma from each of these tubes were immediately frozen (liquid N₂) and stored at -80°C prior to processing and analysis. Aliquots from each of the Li/Hep tubes were used as analytical replicates.

Urine was collected into suitable containers and stored under refrigerated conditions (or in a cool bag with cool packs when subjects were not on site). Following the final collection, volumes were recorded and aliquots were taken and frozen. Samples were stored at -80°C prior to processing and analysis.

2.5 Clinical Biochemistry measures

Plasma glucose, triglycerides, total cholesterol, HDL and LDL cholesterol were measured from 1 ml Li/Hep aliquots by standard enzymatic assays on a clinical analyser at the Core Biochemical Assay Laboratory (CBAL) at Addenbrooke's Hospital, Cambridge. Insulin and

C-peptide were measured from the same 1 ml aliquots by immunoassay by the CBAL. Urinary creatinine and the electrolytes sodium, potassium and chloride were measured in reconstructed 24 h urine collections from the study day by standard assays and also by the CBAL.

2.6 Metabolomic sample preparation

Biological samples were prepared as previously described [21, 22]. Briefly, plasma (200 μ L) was extracted in a 2.0 mL glass beads-containing Eppendorf tube in 1520 μ L at -20 °C pre-chilled methanol/ CHCl_3 (4:1) using a mixer-mill (MM300, Retsch) for 30 sec at 30 Hz, vortexed, stored at -20 °C in a freezer for 20 minutes, shaken for 15 min at 4 °C and then centrifuged for 6 min at 4 °C and 13,000 rpm (Biofuge fresco, Hareaus). For FIE-MS and GC-tof-MS analysis 100 μ L and 420 μ L supernatant was dried respectively in vacuo (speedvac, Univapo). Urine samples (50 μ L) were diluted with 450 μ L of pre-chilled methanol/water (3.5:1), vortexed and shaken and centrifuged as for plasma. For GC-tof-MS analysis 300 μ L supernatant was dried in the speedvac and derivatised according to [21, 22]. Extracts and dried samples were stored at -80 °C until required. For FIE-MS analysis, plasma samples were reconstituted in 100 μ L Methanol/ H_2O 70/30, urine samples were used directly.

2.7 Metabolite fingerprinting and profiling

Urine and plasma samples were analyzed separately in randomized batches. All biological samples of a biofluid were analyzed in a hierarchical approach as reported previously [21]. FIE-MS fingerprinting on an LTQ linear ion trap (Thermo Electron Corporation) was followed by in-depth uni- and multivariate supervised and non-supervised data analysis and feature selection tools using the FIEMspro workflow (R-package available at: <http://users.aber.ac.uk/jhd/>) [25] as described in detail elsewhere [22]. The identity of potential discriminatory ions (m/z) was investigated by analysis of selected nominal mass bins in pooled samples using targeted nano-flow Fourier-Transform ion cyclotron resonance Mass Spectrometry (FT-icr-MS) using a TriVersa NanoMate (Advion BioSciences Ltd) on a LTQ-FT-ULTRA (Thermo Scientific) to obtain ultra-high accurate mass information and MS n ion-trees [22]. These accurate masses were subsequently used to search the LC-MS annotation database MZedDB. Metabolite profiling by GC-tof-MS was undertaken to identify further sucrose responsive metabolites not detected well by FIE-MS and to confirm results of

putative assignments. GC-tof-MS profiling on Pegasus III (Leco Inc) and subsequent data analysis was carried out as reported recently [22].

2.8 Statistical analysis

To test for the effects of time and treatment on clinical markers either 1-way or 2-way Analysis of Variance (ANOVA) was used depending on the appropriate test required. Pearsons correlations between variables from specific time points in each treatment group were also assessed. For metabolite fingerprinting analysis samples were subjected to unsupervised and supervised multivariate classification using Principal Components Analysis (PCA) and PC-Linear Discriminant Analysis (PC-LDA), respectively. Classification and data mining was carried out by following the FIEmSpro workflow validated previously by Aberystwyth [25] (<http://users.aber.ac.uk/jhd/>). Eigenvalues (Tw), Classification accuracies and Random Forest (RF) ‘margins’ between data models were calculated to provide metrics of similarity. Classification accuracies in excess of 95% with model margins > 0.2 or Eigenvalues > 1.5 in such pair-wise comparisons were taken as evidence of adequate differences. Feature selection techniques were used as described previously [26] to highlight the potentially explanatory mass signals responsible for discriminating between different dietary exposure level classes. For GC-tof-MS data filtering purposes metabolite signals with $P > 1 \times 10^{-4}$ following all pair-wise comparisons of the different sample classes were removed from the data set.

3. Results

3.1 Volunteer baseline characteristics and dietary analysis

Table 1 shows the subject characteristics at baseline prior to the sucrose intervention. There were no significant differences between any of the variables between groups. There was a relatively low SD in age, BMI, average sucrose intake and total carbohydrate intake, and normal electrolyte excretion, glucose control and lipid status parameters.

3.2 FIE-MS sample classification to detect metabolites associated with acute sucrose exposure

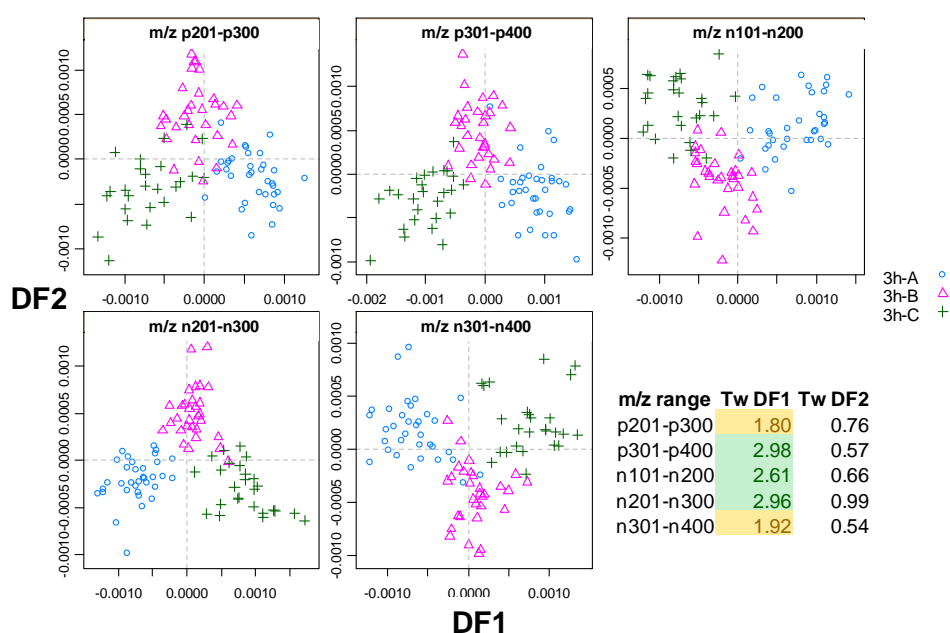
Principal Components Linear Discriminant Analysis (PC-LDA) was performed on FIE-MS data in 100 m/z ranges. Class discrimination was visualized in PC-LDA score plots, with Tw values of the adequate models in urine (**Fig. 1A**) and plasma (**Fig. 1B**), showing discrimination between the three treatment groups. In urine, discrimination was strongest for m/z-ranges 201-400 in positive and 101-400 in negative ionisation mode at the 3 h time-point (Tw >2), with poor models (Tw <1.5) found in all other time points. In plasma, discrimination was strongest for m/z-ranges 101-400 in positive and 101-400 in negative ionisation mode also at the 3 h time-point. Only these models were pursued further in terms of feature selection.

Table 1. Baseline characteristics and dietary intake of all subjects

	Total group	Treatment group		
		0 g	50 g	100 g
Volunteers [n]	97	35	31	31
Age [y]	29.9 ± 4.7	29.8 ± 4.4	30.5 ± 5.2	29.1 ± 4.1
BMI [Kg/m ²]	23.3 ± 2.5	23.1 ± 5.4	23.1 ± 2.3	23.6 ± 2.9
Body fat [%]	30.1 ± 5.3	29.6 ± 5.1	30.2 ± 5.7	30.4 ± 5.1
Systolic BP [mmHg]	115 ± 12	113 ± 12	113 ± 11	118 ± 11
Diastolic BP [mmHg]	70 ± 7	72 ± 8	69 ± 7	69 ± 7
<i>Dietary intake:</i>				
Energy [kcal]	1854 ± 427	1838 ± 410	1849 ± 410	1875 ± 474
Protein [g]	75 ± 20	74 ± 18	74 ± 19	76 ± 22
Fat [g]	73 ± 24	71 ± 26	75 ± 23	75 ± 23
Alcohol [g]	11 ± 17	9.6 ± 20.5	12 ± 15	10 ± 16
Carbohydrate [g]	204 ± 50	209 ± 49	198 ± 45	206 ± 58
Total sugars [g]	86 ± 31	84 ± 29	82 ± 26	93 ± 37
Sucrose [g]	35 ± 14	35 ± 13	31 ± 13	38 ± 17
Fructose [g]	18 ± 9	16 ± 7	18 ± 11	19 ± 10
Glucose [g]	16 ± 8	15 ± 6	16 ± 10	16 ± 8
<i>Electrolytes:</i>				
Na ⁺ [mmol/L]	44.4 ± 19.1	47.3 ± 19.2	42.8 ± 19.8	42.5 ± 18.5
K ⁺ [mmol/L]	22.0 ± 8.6	23.3 ± 6.9	19.1 ± 8.3	23.4 ± 10.1
Cl ⁻ [mmol/L]	48.5 ± 19.6	50.7 ± 19.1	45.6 ± 19.2	48.9 ± 20.8
Creatinine [mmol/L]	5.8 ± 2.4	6.11 ± 2.3	5.0 ± 2.3	6.4 ± 2.6
<i>Glucose control:</i>				
HOMA [IR]	7.3 ± 3.6	7.6 ± 3.7	7.0 ± 2.9	7.5 ± 4.1
HOMA [%B]	489 ± 217	481 ± 225	535 ± 223	449 ± 181
Glucose [mmol/L]	4.89 ± 0.3	4.96 ± 0.4	4.75 ± 0.3	4.95 ± 0.3
Insulin [pmol/L]	33 ± 15	34 ± 16	32 ± 13	33 ± 17
<i>Lipid status:</i>				
Triglycerides [mmol/L]	0.84 ± 0.30	0.79 ± 0.33	0.81 ± 0.29	0.91 ± 0.26
Total Cholesterol [mmol/L]	4.53 ± 0.70	4.51 ± 0.74	4.47 ± 0.65	4.62 ± 0.72

LDL-Cholesterol [mmol/L]	1.76 ± 0.32	2.33 ± 0.49	2.39 ± 0.47	2.45 ± 0.58
HDL-Cholesterol [mmol/L]	2.39 ± 0.51	1.80 ± 0.39	1.72 ± 0.29	1.75 ± 0.27

A Urine



B Plasma

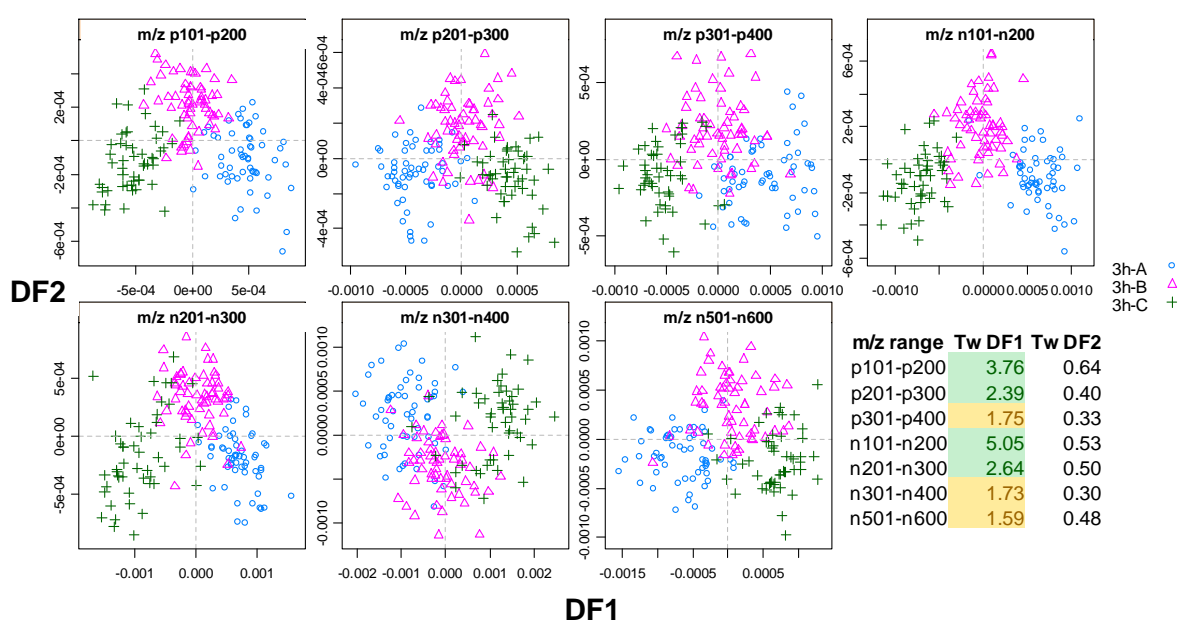


Figure 1. Comparison of sucrose treatment groups by Flow-Injection Electrospray Mass Spectrometry (FIE-MS) fingerprinting of 3 h (a) urine and (b) plasma samples: Principal Components Linear Discriminant Analysis score plots of Discriminant Function 1 (DF1) and DF2 for informative FIE-MS mass ranges in both positive (p) and negative (n) ionization modes. Subjects grouped as A (0 g sucrose, control), B (50 g sucrose) and C (100 g sucrose).

3.3 Selection of metabolome features explanatory of acute sucrose exposure

RF importance scores highlighted around 120 important m/z values in both plasma and urine that differed in intensity between treatment groups. From this list the top ranked variables in urine (16 metabolites) and plasma (24 metabolites) underwent putative identification and correlation analysis to give more insight into trends of metabolite behaviour as a response to the different levels of sucrose treatment.

Correlation analysis of these major discriminatory ions are shown in **Figure 2A** and **B** for urine and plasma respectively. In urine the features are split between those that are positively and negatively correlated with sucrose intake (**Figure 2A**). The variable m/z n215.18 was the overall strongest discriminator (Rank 1), but only important in 3 h data whereas other variables were discriminatory at two consecutive time points (3 h and 6 h e.g. m/z n135.18, p381) and were highly correlated. The highest ranked negatively correlated ion was m/z n139.18 which was highly correlated with m/z n161.18 and n101.18 but the latter were less highly ranked than the other variables in urine.

In plasma, signals are split into two main clusters (**Figure 2B**): one with a strong positive correlation with sucrose intake (Clade 1) and a second divided into different levels of negative correlation with sucrose intake (Clades 2 to 4). The most highly ranked signal that was positively correlated with sucrose intake was m/z n263.18 and n207.18 that were highly correlated. The most highly ranked variables were negatively correlated with sucrose intake and appeared in Clade 4 (e.g. m/z n130.27, p154.18, n129.27).

3.4 Annotation of discriminatory signals and relative intensities with sucrose intake

Signals suggested by the MZedDB were confirmed using GC-tof-MS and comparison with standards. Some of the FIE-MS nominal mass bins contained up to 10 accurate mass ions in FT-icr-MS spectra, some of which did not result in a hit from MZedDB. However, the list of discriminatory variables was dominated by organic acid or fatty acids isomer putative assignments. Many of the explanatory plasma signals were highly correlated and so are grouped into Clades in **Table 2** which helps to determine signal relationships. For example, whereas metabolites in Clade 2 were mainly free fatty acids, Clade 3 contains the main ketone bodies, acetoacetone and 3-hydroxybutanoic acid. Clade 4 contains metabolites associated with branched chain amino acids metabolism. Several signals were found highly discriminatory in both the urine and plasma data. These included n101.27 (acetoacetate), and n139.27 (octadienoic acid), both negatively correlated with sucrose intake (**Figure 2**).

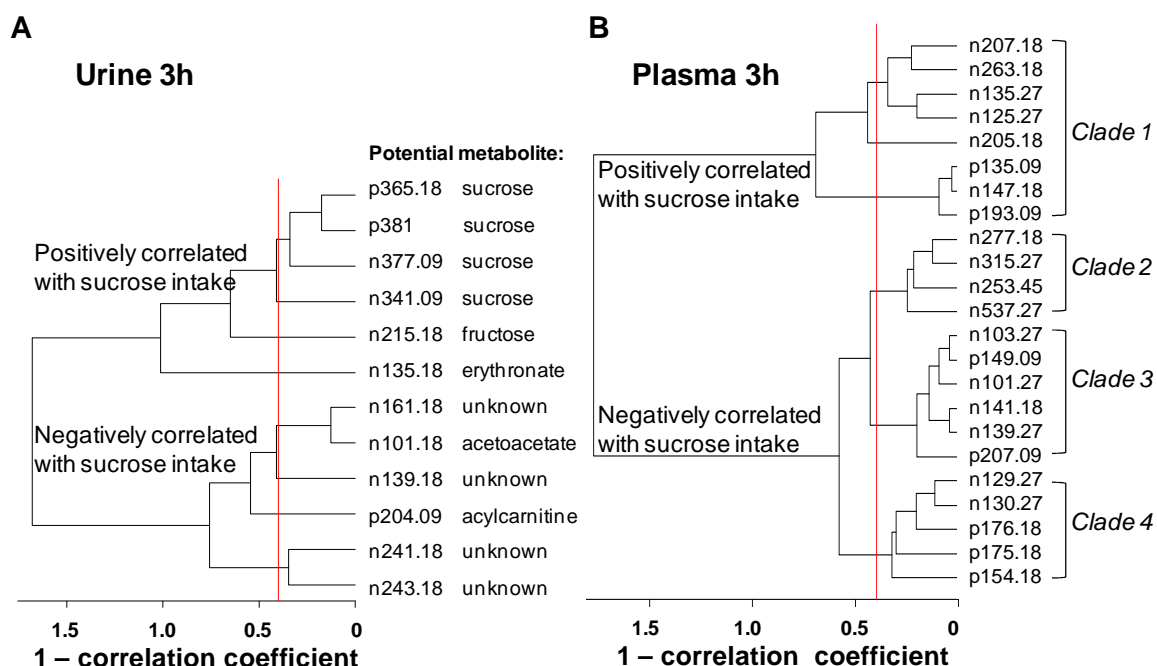


Figure 2. Hierarchical cluster analysis of Log10-transformed and TIC-normalized intensity values of putative metabolites discriminating between sucrose treatments in (A) 3 h urine (B) 3 h plasma samples with ions representing positive (p) and negative (n) ionisation mode features.

The signals n135.18 (urine) and n135.27 (plasma) that were positively correlated with sucrose intake (**Figure 2**) were assigned as erythronic acid. The identity of erythronic acid was confirmed by comparing retention time characteristics of erythronic acid standards with threonic acid with those observed in plasma GC-tof-MS data and those reported in the Golm Metabolite Data base [27].

Examples of relative intensity distributions of highly ranked variables between particular treatment groups and specific time points in urine data are shown in **Figure 3**. The left panel shows three signals most strongly positively correlated with sucrose intake. The chloride adduct of fructose was only explanatory in 3 h urine samples, whereas the potassium adduct of sucrose was discriminatory at the 6 h time point. The signal intensities of negatively correlated explanatory mass ions show the opposite trend (right panel in **Figure 3**); with metabolites found at higher levels in urines of those volunteers in the 0 g sucrose group. In the examples shown (hydroxybutanoic acid acid, acetyl carnitine, and tridecanedionic acid)

the differences between treatment groups were highly significant still at 9 h. In these examples the highest sucrose dose showed the least increase in metabolite levels.

Examples of relative intensity distributions of highly ranked variables in plasma data are shown in **Figure 4**. The left hand panel shows the levels of three example m/z ions which demonstrated a positive correlation with sucrose treatment from Clade 1 (**Table 2**). Intensities of these positively correlated metabolites were significantly increased at the 3 h time point in the 50 g and 100 g groups, and the intensity had returned to baseline after 24 h.

The opposite trend was seen in those signals negatively correlated with sucrose intake, examples of which are shown in the right panel of **Figure 4**. In these signals from Clade 4 (**Table 2**) the intensity at 3 h was lower than at baseline in the 50 g and 100 g groups, however in the 0 g group intensities appeared to increase up to 24 h later and significant effects were observed at both 3 h and 24 h.

Table 2. Tentative identity of highly ranked urine and plasma signals potentially explanatory of acute dietary sucrose exposure using Fourier Transform-Ion Cyclotron Resonance Ultra-Mass-Spectrometry (FT-icr-MS). Flow-Injection Electrospray Mass Spectrometry (FIE-MS) mass ions are listed in the order as shown in Figure 2A and 2B, respectively. Listed are only those accurate masses which resulted a hit in MZedDB. The dominant accurate mass ion is printed in bold.

Rank [§]	FIE-MS	FT-MS[m/z]	Adduct	– Adduct	Potential Metabolites*
Urine					
Positively correlated					
4	p365.18	365.10544	C12H22NaO11	[M+Na] ¹⁺	Sucrose* (and other disaccharides)
2	p381	381.07937	C12H22KO11	[M+K] ¹⁺	Sucrose* (and other disaccharides)
12	n377.09	377.08562	C12H22ClO11	[M+Cl] ¹⁻	Sucrose* (and other disaccharides)
7	n341.09	341.10894	C12H21O11	[M-H] ¹⁻	Sucrose* (and other disaccharides)
1	n215.18	215.03288	C6H12ClO6	[M+Cl] ¹⁻	Fructose* (and hexose isomers)
6	n135.18	135.03003	C4H7O5	[M-H] ¹⁻	Erythronic acid*
Negatively correlated					
16	n161.18	161.04567	C6H9O5	[M-H] ¹⁻	Hydroxyl-hexanedioic acid
14	n101.18	101.02448	C4H5O3	[M-H] ¹⁻	Acetoacetate (and isomers)
3	n139.18	139.07655	C8H11O2	[M-H] ¹⁻	Octadienoic acid (and isomers)
		139.01676	C4H8ClO3	[M+Cl] ¹⁻	3-Hydroxybutanoic acid*
10	p204.09	204.12320	C9H18NO4	[M+H] ¹⁺	Acetylcarnitine
			C9H18NO4	[M ¹⁺] ¹⁺	O-Acetylcarnitine
13	n241.18	241.11936	C11H17N2O4	[M-H] ¹⁻	Unknown
		241.21728	C15H29O2	[M-H] ¹⁻	Pentadecanoic acid (and isomers)
11	n243.18	243.06224	C9H11N2O6	[M-H] ¹⁻	Uridine
		243.13501	C11H19N2O4	[M-H] ¹⁻	Unknown

Rank [§]	FIE-MS	FT-MS[m/z]	Adduct	– Adduct	Potential Metabolites*
Plasma					
Positively correlated Clade 1					
7	n207.18	207.07939	C9H16ClO3	[M+Cl] ¹⁻	Keto-nonanoic acid (and isomers)
		207.11582	C10H20ClO2	[M+Cl] ¹⁻	Decanoic acid*
6	n263.18	263.12879	C15H19O4	[M-H] ¹⁻	Unknown
23	n135.27	135.02999	C4H7O5	[M-H] ¹⁻	Erythronic acid*
14	n125.27	125.00113	C3H6ClO3	[M+Cl] ¹⁻	Lactic acid
8	n205.18	205.14464	C10H21O4	[M-H] ¹⁻	Unknown
		205.06371	C9H14ClO3	[M+Cl] ¹⁻	2-Butyl-4-hydroxy-5-methyl-3(2H)-
22	p135.09	135.08043	C9H11O	[M+H-FA] ¹⁺	10-hydroxy-2E,8Z-Decadiene-4,6-
15	n147.18	147.02999	C5H7O5	[M-H] ¹⁻	Methylmalic acid
		147.06637	C6H11O4	[M-H] ¹⁻	2,3-Dihydroxy-3-methylpentanoate
21	p193.09	193.12240	C12H17O2	[M+H] ¹⁺	Dodecatetraenoic acid
		193.14351	C9H21O4	[M+H] ¹⁺	Unknown
Negatively correlated Clade 2					
18	n277.18	277.18087	C17H25O3	[M-H] ¹⁻	Hydroxy-heptadecadien-ynoic acid;
		277.21722	C18H29O2	[M-H] ¹⁻	Octadecatrienoic acid; linolenic acid
10	n315.27	315.20956	C18H32ClO2	[M+Cl] ¹⁻	Octadecadienoic acid
		315.25398	C18H35O4	[M-H] ¹⁻	Dihydroxy-octadecanoic acid
9	n253.45	253.21720	C16H29O2	[M-H] ¹⁻	Hexadecenoic acid
13	n537.27	537.04643			No hit
Negatively correlated Clade 3					
17	n103.27	103.04011	C4H7O3	[M-H] ¹⁻	3-Hydroxybutanoic acid*
4	p149.09	149.11726	C7H17O3	[M+H] ¹⁺	Unknown
24	n101.27	101.02448	C4H5O3	[M-H] ¹⁻	Acetoacetate* (and isomers);
16	n141.18	141.01704	C4H6NaO4	[M+Na-2H] ¹⁻	Dihydroxybutanoic acid*
11	n139.27	139.07655	C8H11O2	[M-H] ¹⁻	Octadienoic acid (and isomers)
		139.01676	C4H8ClO3	[M+Cl] ¹⁻	3-Hydroxybutanoic acid*
12	p207.09	207.17449	C14H23O	[M+H-FA] ¹⁺	Tetradecadienoic acid
Negatively correlated Clade 4					
3	n129.27	129.05579	C6H9O3	[M-H] ¹⁻	3-Methyl-2-oxopentanoic acid
1	n130.27	130.08744	C6H12NO2	[M-H] ¹⁻	Iso-leucine (and isomers)
19	p176.18	176.10706	C11H14NO	[M+H] ¹⁺	Unknown
5	p175.18	175.09654	C8H15O4	[M+H] ¹⁺	Octenedioic acid (and isomers)
		175.13291	C9H19O3	[M+H] ¹⁺	Hydroxyl nonanoic acid
2	p154.18	154.08379	C6H13NNaO2	[M+Na] ¹⁺	Iso-leucine (and isomers)
		154.05865	C4H9N3NaO2	[M+Na] ¹⁺	Creatine

[#] Metabolites found in the same correlation cluster are grouped by clade (Figure 2).

* Identity of discriminatory metabolites confirmed by GC-tof-MS analysis.

[§] Rank based on Random Forest importance scores.

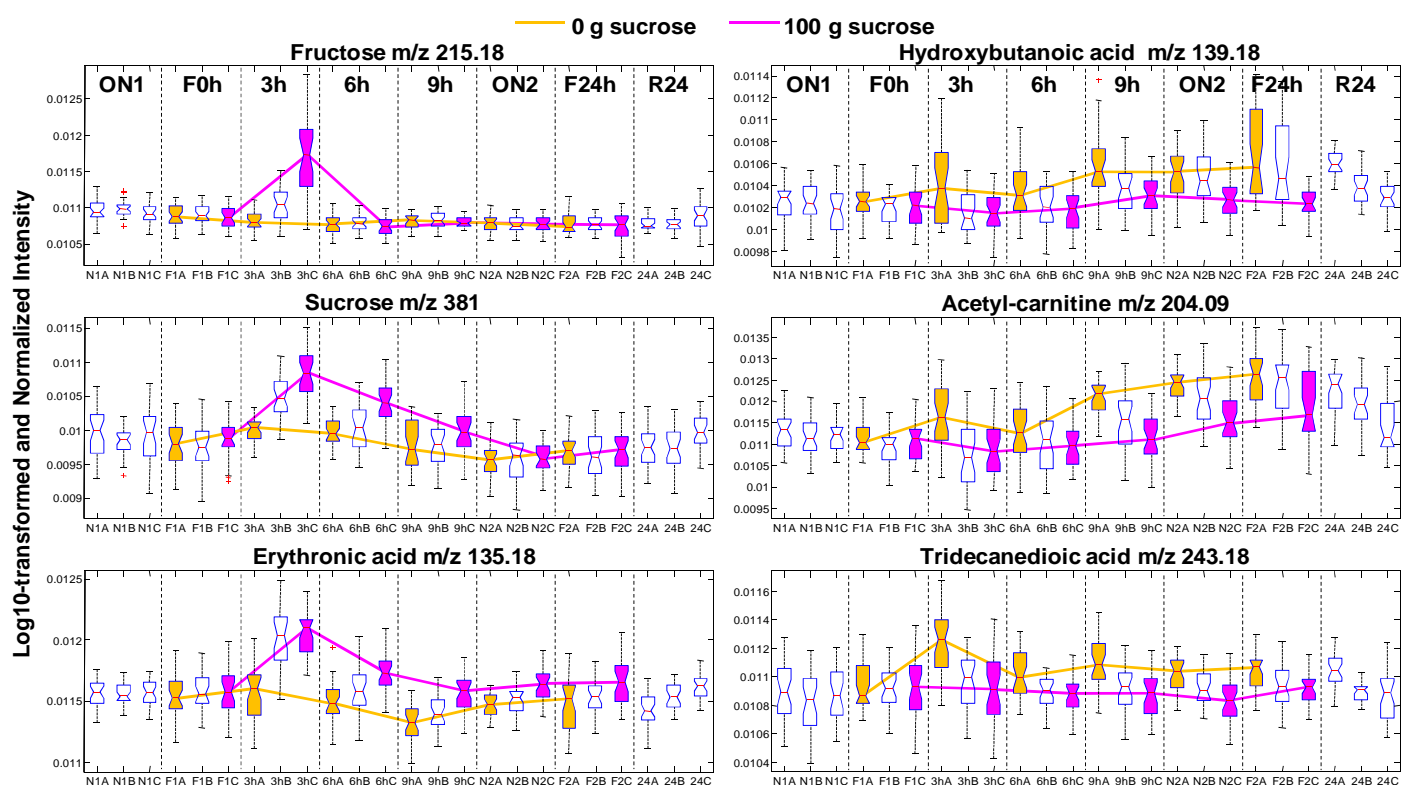


Figure 3. Examples of Log10-transformed and TIC-normalised intensity values of m/z variables that discriminate for sucrose treatment in urine samples: repartitioning of urine FIE-MS data by meta-class sampling time and treatment. Left panel, positively correlated with sucrose intake; right panel, negatively correlated with sucrose intake. Sampling times post sucrose intake are shown; ON, overnight urine collections; F, fasting; R, reconstituted 24 h urine sample.

3.5 GC-tof-MS profiling

PC-LDA of urine GC-tof-MS data showed very good discrimination ($DF1$, $Tw=9$) between treatment at the 3 h time point, but in plasma, the 3 h time point provided only an adequate model ($DF1$, $Tw=3.65$). In urine, the list of explanatory metabolites was extremely short and the intensity profiles of the 4 main discriminatory metabolites are shown in **Figure 5**. At the 3 h time point sucrose and fructose strongly discriminate treatment groups in urine samples, being positively correlated with sucrose intake. Sucrose was still explanatory for treatment differences in 9 h samples. The metabolites 3-amino-2-methyl-propanoic acid and 3-hydroxybutanoic acid showed the opposite trend as the intensities consistently remained lower in the 100 g group and negatively correlated with sucrose intake at 3 h.

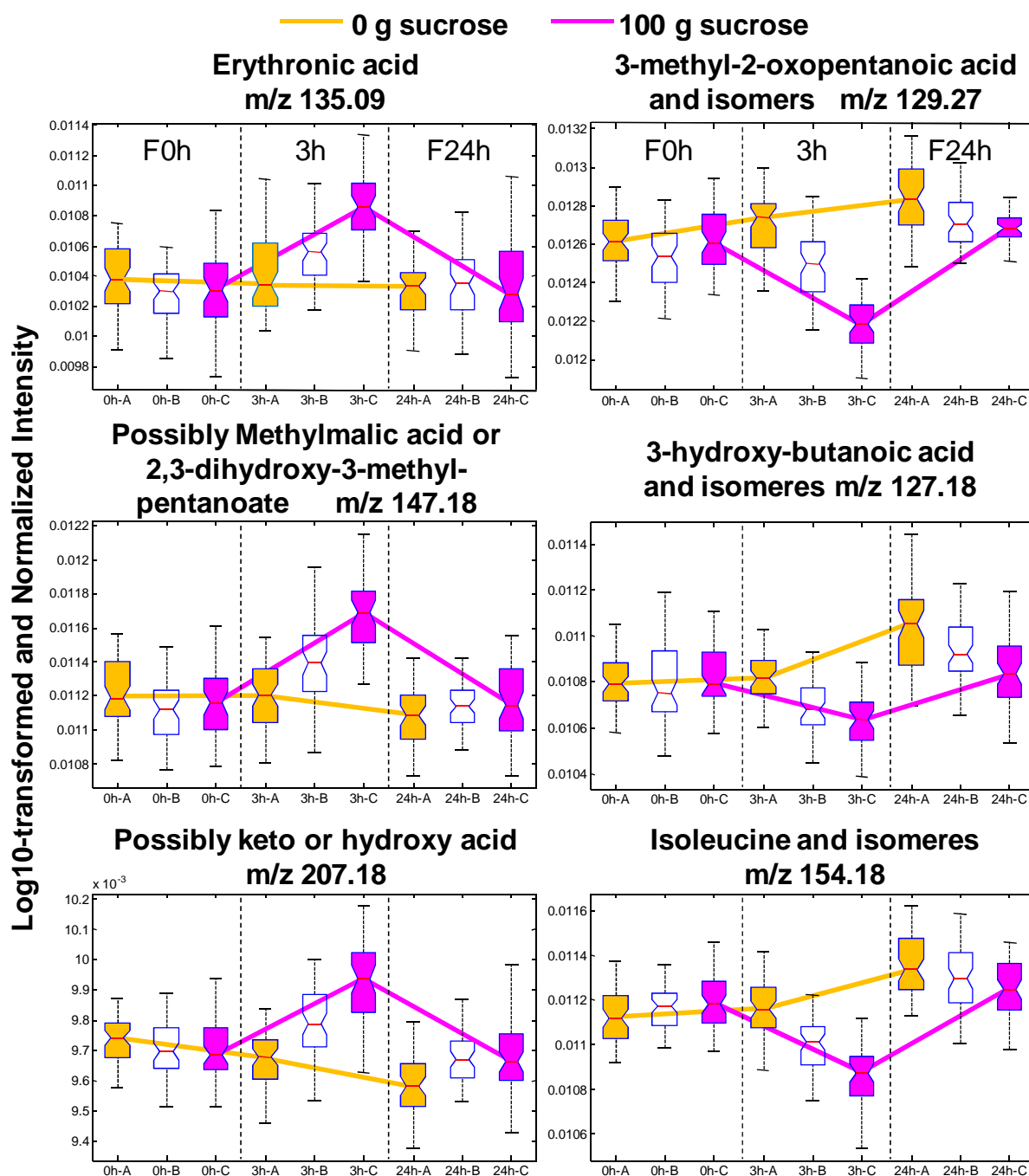


Figure 4. Examples of Log10-transformed and TIC-normalised intensity values of m/z variables that discriminate for sucrose treatment in plasma samples: repartitioning of urine FIE-MS data by meta-class sampling time and treatment. Left panel, positively correlated with sucrose intake; right panel, negatively correlated with sucrose intake. Sampling times post sucrose intake are shown; F, fasting.

In plasma (data not shown), results mirrored that from FIE-MS in that sucrose, fructose, erythronic acid and one unknown metabolite (characterised by m/z 163, 214, 233, 304), were

positively correlated with sucrose intake while metabolites including acetoacetic acid, two hydroxy-butanoic acid isomers, 3-amino-2-methyl-propanic acid and eight free fatty acids (saturated and unsaturated), were negatively correlated with sucrose intake and showed similar relative intensity distributions.

4. Discussion

In this study we have investigated the influence of acute sucrose intake on urinary and plasma metabolite profiles using metabolomics with the aim of understanding the metabolic impact of acute sucrose intake and identifying metabolites with the potential to be developed into biomarkers of sucrose intake. This study is novel in that it is the first to use a range of non-targeted MS-based technologies and the first to examine both urine and plasma metabolomes following acute exposure to sucrose at intakes commonly observed in the average UK population. We demonstrate here that sucrose ingestion has influenced metabolism with over 120 metabolites in both urine and plasma that discriminated between sucrose treatments resulting in differing responses to sucrose intake. We have shown that a number of metabolites are directly related to sucrose intake; specific metabolites positively correlated to sucrose intake and that have not been previously identified could be developed further into biomarkers, metabolites that are increased in intensity but not correlated to sucrose intake appear to be in response to the ingestion of water, and those that are negatively correlated to sucrose intake and dominant in the control group appear to be due to an overall fasting response and metabolic switch from fasting to the fed state.

4.1 Influence of sucrose intake on metabolism

As would be expected [13, 28, 29], we found sucrose and fructose as responsive features in both plasma and urine (Figures 3 & 4). Sucrose is commonly found in the urine of individuals due to the inefficiency of sucrase enzymatic cleavage. It is thought that the majority of plasma sucrose is excreted in the urine [30], however there is little understanding of plasma sucrose kinetics and tissue uptake as the dose-responsive kinetic nature of sucrose/fructose in plasma has not been fully explored in previous studies. The urinary excretion profiles of sucrose and fructose were different. Fructose demonstrated a maximum excretion only at 3h, whereas for sucrose raised levels were evident at extended time points (6h and 9h post).

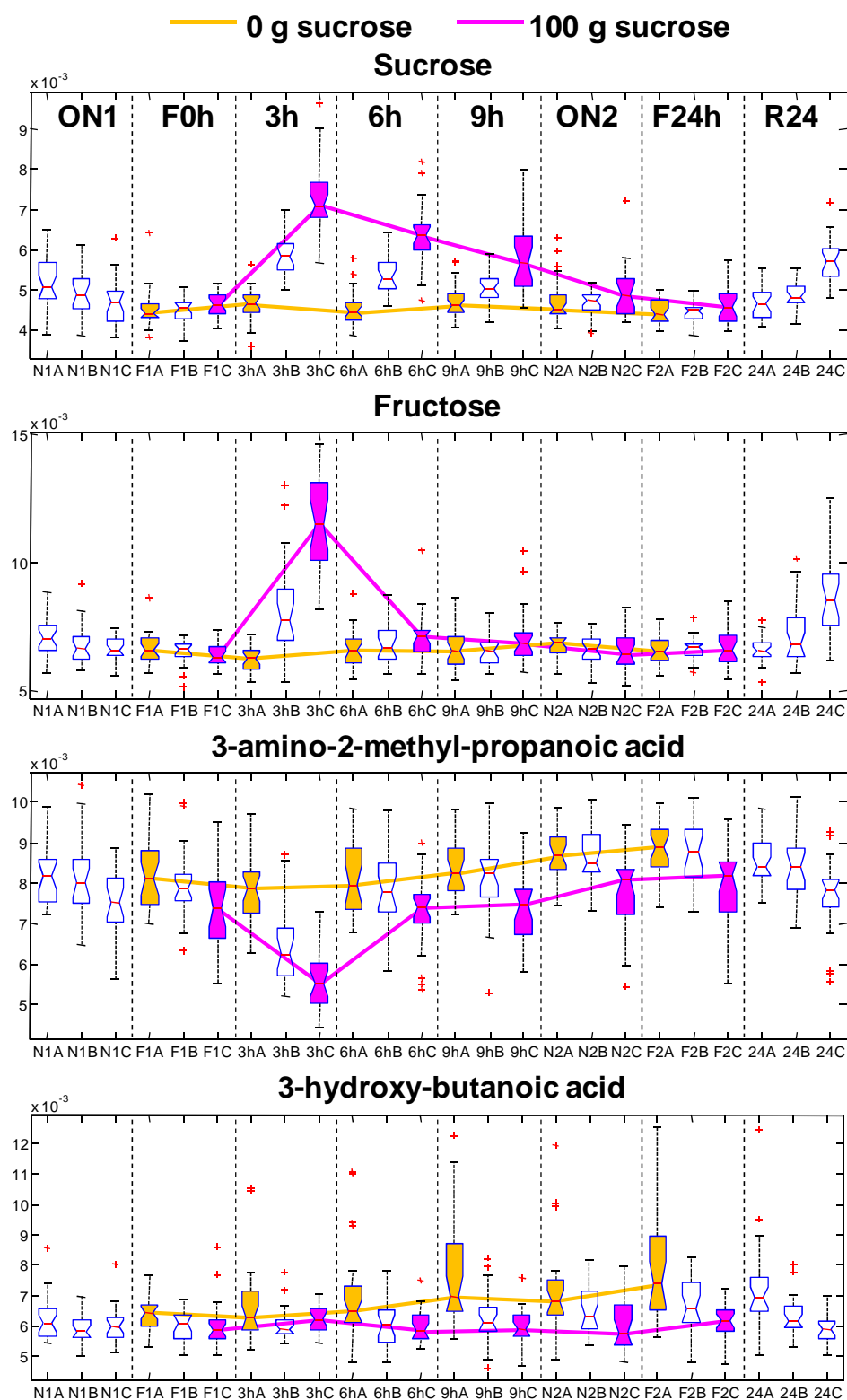


Figure 5. Log10-transformed intensity values of sucrose, fructose, 3-amino-2-methyl-propanoic acid and 3-hydroxy-butanoic acid discriminating between sucrose treatment: repartitioning of urine Gas Chromatography –time-of-flight - Mass Spectrometry (GC-tof-MS) data by meta-class sampling time and treatment. Sampling times post sucrose intake are shown; ON, overnight urine collections; F, fasting; R, reconstituted 24 h urine sample.

This would be expected given the differences in metabolic fate between sucrose and fructose. The rapid removal of fructose from the plasma and the low abundance of fructose in 3h plasma samples highlights rapid fructose metabolism. The pharmacokinetics of plasma fructose following either sucrose or HCFS intake found a Tmax of 60 min and by 180 min the concentration had returned to baseline [29]. The metabolic fate of sucrose in plasma is unknown.. As we observed a more persistent appearance of sucrose in the urine over the course of the day (Figure 3) we can speculate that there is limited transport into cells and sucrose is gradually filtered into the urine. The observation that sucrose and fructose were not as explanatory in plasma compared to urine suggests that sucrose is removed efficiently from blood and excreted; our urine data suggest that sucrose has a half-life of about 3 hours.

Erythronic acid (2,3,4-tri-hydroxybutyric acid) also demonstrated a positive correlation with sucrose intake in both urine and plasma (Figs 3 & 4), and this is the first study to demonstrate this link. This sugar can be formed through Maillard reactions fragmenting sugar moieties [31]. Erythronic acid is normally found in low concentrations in both urine and plasma, but is increased in situations where the flux through the pentose phosphate pathway is disturbed [32]. This would lead to an accumulation of metabolites and flux through alternative pathways that result in the formation of increased amounts of erythronic acid [32]. It is an interesting observation that sucrose intake could potentially cause this effect and one that has not been previously observed in humans and could contribute further to the metabolic dysregulation induced by excess fructose.

Table 3. Summary of fatty acid derivatives negatively correlated with sucrose intake putatively identified in plasma and urine signals from FIE-MS and GCMS.

Carbon backbone	NEFA	Carnitine derivative
C8	C8:1	C8:0
	C8:2	
C9	C9:0	
C10	C10:0	C10:0
C12	C12:4	C12:0
C13	C13:2	
C14		C14:1
C15	C15:0	
C16	C16:1	
C18	C18:1	C18:1
	C18:2	C18:2

There were further signals that were positively correlated with sucrose intake, but were not in the top ranked metabolites investigated further, and these could indicate other metabolic pathways influenced by sucrose. For example, m/z 147.18 in plasma could potentially be a lactone intermediate from the pentose phosphate pathway or methylmalonic acid, an important intermediate in fat and protein metabolism; both of which would align with biochemical pathways shown to be influenced in the current study.

Urinary data over time revealed a number of metabolites with a common profile over time that were increased in intensity during the intervention in all groups indicating that the response was not dependent on sucrose intake, and therefore they were not investigated further. In these examples (data not shown), it is likely that the sudden influx of fluid following fasting caused a release of accumulated metabolites into the plasma and rapid clearance by the kidney.

Plasma and urine data demonstrated a number of metabolites that were negatively correlated to sucrose intake and were increased in intensity in the control group that received only water, but were lower in groups ingesting sucrose (Figs 3 & 4). This group of metabolites include a range of free fatty acids, acyl-carnitines and ketone bodies and represent classical markers of a fasting response. This fasting response is somewhat understandable given the study design, in that subjects in the control group received zero calories until their standard lunch (496 kcal), 4-5 hours following the ingestion of sucrose/water.

From FIE-MS and GC-tof-MS analysis we found fatty acyl moieties, either NEFA or carnitine derivatives, from 10 separate carbon backbones ranging from medium (C8) to long chain (C18) and a mix of saturated and unsaturated (**Table 3**). NEFA have previously been shown to be raised during the fasting state, in which fatty acids are mobilised to act as an energy source via β -oxidation [33]. It has been shown that fructose intake has a lipogenic effect and raises plasma triglycerides [5], however we did not observe such lipogenic effects in the present study. Therefore an acute dose of up to 100 g sucrose does not increase NEFA or triglycerides in the 24 h timeframe of the current study, and this is an important observation. We observed at least 5 differing acyl (fatty acid)-carnitine moieties with varying fatty acid chain length and saturation negatively correlated to sucrose intake (Table 3). Acyl-carnitines, substrates for fatty acid β -oxidation, are present during fasting [33] and have also been observed during an OGTT [10, 11]. Ketone bodies such as 2-hydroxybutyrate, 3-

hydroxybutyrate, acetoacetate and acetone have been found as biomarkers of fasting in a metabolomic approach [33] and shown to decrease in response to glucose [11]. In the current study we observed ketone bodies increasing in intensity with time during the day and negatively correlated to sucrose intake (e.g 3-hydroxybutanoic acid in urine and acetoacetate in plasma, Fig 3). We also found leucine/isoleucine as a discriminatory metabolite in plasma and 3-amino-2-methyl-propanoic acid in urine that were negatively correlated with sucrose intake. Amino acids and branched chain amino acids, such as leucine/isoleucine and keto-amino acid derivatives, have been identified as novel biomarkers of fasting [33]. Intensities of these amino acids were raised in baseline samples, and increased during the day in the control group that received no sucrose.

Although this fasting effect is not directly related to sucrose intake it is interesting that we can still observe dose-responsive relationships to sucrose ingestion, and this finding that differing aspects of the fasting response are related to the level of sucrose intake is novel. For example the observation that ketone bodies and acyl-carnitines were not increased in the groups receiving sucrose indicates that 50 g sucrose (200 kcal) was sufficient to prevent ketosis and mitochondrial fatty acid oxidation. It is also interesting to note that some fasting markers continued to increase in intensity during the study day (e.g Figure 3; acetyl-carnitine, hydroxybutanoic acid) while NEFA were increased only after 3 h (e.g Figure 3; tridecanedioic acid). These observations are of interest in determining the amount of calories required to prevent certain metabolic pathways involved in the fasting response but require further work to characterise.

4.2 Putative biomarkers of sucrose intake.

As we have mentioned, those metabolites that are positively correlated to sucrose intake are candidates as biomarkers of sucrose intake. Urinary sucrose and fructose, which were the highest ranked variables, have been previously suggested as predictive biomarkers for sucrose intake [13, 16]. Although a clear relationship between sucrose intake and urinary fructose/sucrose existed in various studies [12, 13], the authors highlight that it is not clear if these biomarkers could be used to assess sugar intake in larger population studies, and other biomarkers are required. The most promising biomarker candidate from the present study is erythronic acid, which has not previously been linked to sucrose intake. This was in the top ranked variables, positively correlated to sucrose intake, and dose-responsive in urine and plasma. We suggest that erythronic acid, alone or in combination with urinary fructose and/or

sucrose, could be developed further into a biomarker for sucrose intake, but further studies are essential to validate and substantiate these findings.

In summary, we have demonstrated that specific metabolites can be used to follow metabolic changes due to acute sucrose intake in metabolite profiles of plasma and urine, many of which have not been observed previously. It is clear from the type of metabolites correlated with sucrose intake that a number of biochemical pathways were influenced in our intervention but it is difficult to completely understand the metabolic effects of sucrose when various metabolic changes are occurring simultaneously by different stimuli in the same intervention. We have shown metabolic changes due to the onset of a switch in metabolism as a response to the overnight fast coupled with consuming only water, in the presence of NEFA, acyl-carnitines and ketone bodies. We suggest that the metabolites that are positively correlated to sucrose intake such as erythronic acid, fructose and sucrose, alone or in combination, could be investigated further for diagnostic potential as biological markers for sucrose intake.

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Conflict of Interest

The authors declare no conflicts of interest.

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